dramatically dropped when the temperature passed 52°C. See Figure 19B. A first derivative of the signal showed a melting temperature of 55°C, which corresponds with the temperature seen for the oligonucleotide-nanoparticle conjugates and linking oligonucleotides hybridized in solution. See Figure 19B.

Example 11: Assay of a Polyribonucleotide Using Nanoparticle-Oligonucleotide Conjugates as Probes

The previous Examples utilized oligo-deoxyribonucleotides as targets in the assays. The present example demonstrates that the nanoparticle-oligonucleotide conjugates can also be used as probes in assaying a polyribonucleotide. The experiment was carried out by adding 1 µL of a solution of poly(rA) (0.004 A_{260} Units) to 100 µL of gold nanoparticles (~ 10 nM in particles) conjugated to dT_{20} (a 20-mer oligonucleotide containing thymidylate residues) through a mercaptoalkyl linker at the 5'-terminus. The conjugation procedure was that described in Example 3. Following freezing in a Dry Ice/isopropyl alcohol bath, thawing at room temperature, and spotting on a C18 TLC plate as described in Example 4, a blue spot characteristic of aggregation of the nanoparticles by hybridization was observed. Control experiments carried out in absence of the target gave a pink spot, rather than a blue spot.

Example 12: Assay for Protective Antigen DNA Segment of Anthrax Using Nanoparticle-Oligonucleotide Conjugates

In many cases amplification of a double-stranded DNA target by PCR is needed to provided sufficient material for an assay. The present example demonstrates that the nanoparticle-oligonucleotide conjugates can be used to assay for a DNA strand in the presence of its complement (*i.e.*, assaying for a single strand after thermal dehybridization of a double-stranded target) and can recognize and specifically bind to an amplicon obtained from a PCR reaction.

A PCR solution containing a 141 base pair duplex amplicon of the Protective Antigen segment of Anthrax was provided by the Navy (sequence given in Figure 23). The assay for this amplicon was carried out by isolating the DNA from 100 μL of the PCR solution using a Qiaquick Nucleotide Removal Kit (Qiagen, Inc., Santa Clarita, CA) and the standard protocol for this kit, with the exception that elution of the DNA was effected with 10 mM phosphate buffer at pH 8.5, rather than with the buffer provided with the kit. The eluant was then evaporated to dryness on a Speed Vac (Savant). To this residue was added 5 μL of a master mix prepared by mixing equal volumes of each of two solutions of two different oligonucleotide-nanoparticle probes (see Figure 23). Each oligonucleotide-nanoparticle probe was prepared as described in Example 3. The solutions of the probes which were combined to form the master mix were prepared by adding 10 μL of 2 M NaCl and 5 μL of oligonucleotide blocker solution (50 pmoles of each Blocker oligonucleotide (see Figure 23 and below) in a 0.3 M NaCl, 10 mM phosphate, pH 7.0., solution) to 5 μL of full-strength (about 10 nM) nanoparticle-oligonucleotide solution. The amplicon-probe mixture was heated to 100°C for 3 minutes, then frozen in a DRY ICE/ethanol bath and allowed to come to room temperature. A small aliquot (2 μL) was spotted on a C18 TLC plate and allowed to dry. A strong blue spot indicative of hybridization was obtained.

Control tests carried out in the same manner in absence of the amplicon target DNA, in the absence of Probe 1, in the absence of Probe 2, or in the absence of the sodium chloride, were all negative, that is, gave a pink spot. Similarly a test carried out using probes 1 and 2 with a PCR amplicon derived from the Lethal Factor segment of Anthrax in place of the Protective Antigen Segment was negative (pink spot). These controls confirmed that both probes were essential, that salt conditions appropriate for hybridization were needed, and that the test was specific for the specified target sequence.

The oligonucleotide Blockers were added to inhibit binding of the second strand of the initial duplex target (*i.e.*, the strand complementary to the target strand) to regions of the target nucleic acid strand outside the segment that binds to the probes (see Figure 23 for sequences), since such binding interferes with binding of the nanoparticle oligonucleotide

probes to the target strand. In this example, the Blocker oligonucleotides were complementary to the single-stranded target in regions not covered by the probes. An alternative scheme is to use blocker oligonucleotides that are complementary to the PCR complementary strand (the strand complementary to the target strand) outside the region that competes with the probe oligonucleotides.

Example 13: Direct assay of PCR Amplicons without isolation <u>of the amplicons from the PCR solution</u>

The procedure described in Example 12 involved separation of the PCR amplicon from the PCR solution before addition of the nanoparticle-oligonucleotide probes. For many purposes it would be desirable to be able to carry out the assay directly in the PCR solution without preliminary isolation of the polynucleotide products. A protocol for such an assay has been developed and is described below. This protocol has been performed successfully with several PCR products derived under standard conditions using a GeneAmp PCR Reagent Kit with Amplitaq DNA polymerase.

To 50 μL of the PCR sample solution, 5 μL of a mixture of two gold nanoparticle-oligonucleotide probes (0.008 A_{520} Units of each) was added, followed by addition of a solution made up from 1 μL of Blocker oligonucleotides (10 pmoles each), 5 μL of 5 M NaCl, and 2 μL of 150 mM $MgCl_2$. This mixture was heated for 2 minutes at 100°C to separate the strands of the duplex target, the tube was immersed directly in a cold bath (*e.g.*, Dry Ice/ethanol) for 2 minutes, then removed, and the solution allowed to thaw at room temperature (the freeze-thaw cycle facilitates hybridization of the probes with the target oligonucleotide). Finally, a few μL of the solution were spotted on a plate (*e.g.*, C18 RP TLC plate, a silica plate, a nylon membrane, etc.). As usual, blue color signifies the presence of the targeted nucleic acid in the PCR solution; a pink color is negative for this target.